The Microbiome Meets Nanotechnology: Opportunities and Challenges in Developing New Diagnostic Devices

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Monitoring of the human microbiome is an emerging area of diagnostics for personalized medicine. Here, the potential of different nanomaterials and nanobiosensing technologies is reviewed for the development of novel diagnostic devices for the detection and measurement of microbiome-related biomarkers. Moreover, the current and future landscape of microbiome-based diagnostics is defined by exploring the advantages and disadvantages of current nanotechnology-based approaches, especially in the context of developing point-of-care (PoC) devices that would meet the international guidelines known as REASSURED (Real-time connectivity; Ease of specimen collection; Affordability; Sensitivity; Specificity; User-friendliness; Rapid & robust operation; Equipment-free; and Deliverability). Finally, the strategies of the latest international scientific consortia working in this field are analyzed, the current microbiome diagnostics market are reported and the principal ethical, legal, and societal issues related to microbiome R&D and innovation are discussed.

1. Introduction

Since the dawn of evolution, microorganisms have co-evolved with every plant and animal species, determining the physiology and health condition of their hosts, including modern humans and our ancestors. As affirmed by Lederberg & McCray nearly 2 decades ago: "the microbiome is an ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space and have been all but ignored as determinants of health and disease".[1] Since then, the paucity of knowledge on the links between human microbiota and human health have been increasingly addressed in academia, the clinic and industry.

1.1. Commensal Microbes: A Necessary and Ever-Changing Organ

A recent study has estimated that in and on the human body, there is a 1:1 ratio of microbial and human cells.[2] These microbial cells form the human microbiome. Although most (60%) microbial cells in humans are located in the gut, others can be found in areas such as the gastrointestinal tract, the skin, the genital surfaces and the oral cavity (**Figure 1**).[3] The interactions between humans and the human microbiome, known as mutualistic symbiosis, has been shaped through millions of years of co-evolution that has strengthened both parties.[4] On one hand, humans provide their commensal microbes with nutrients and shelter; on the other, these microbes assist their human hosts by performing location-specific functions such as immune defense (Figure 1). In fact, the microbiota has together been considered as a separate "organ".[5] However, from birth, said "organ" is constantly evolving, as it is influenced by genetic as well as by environmental factors (e.g., diet, stress, or

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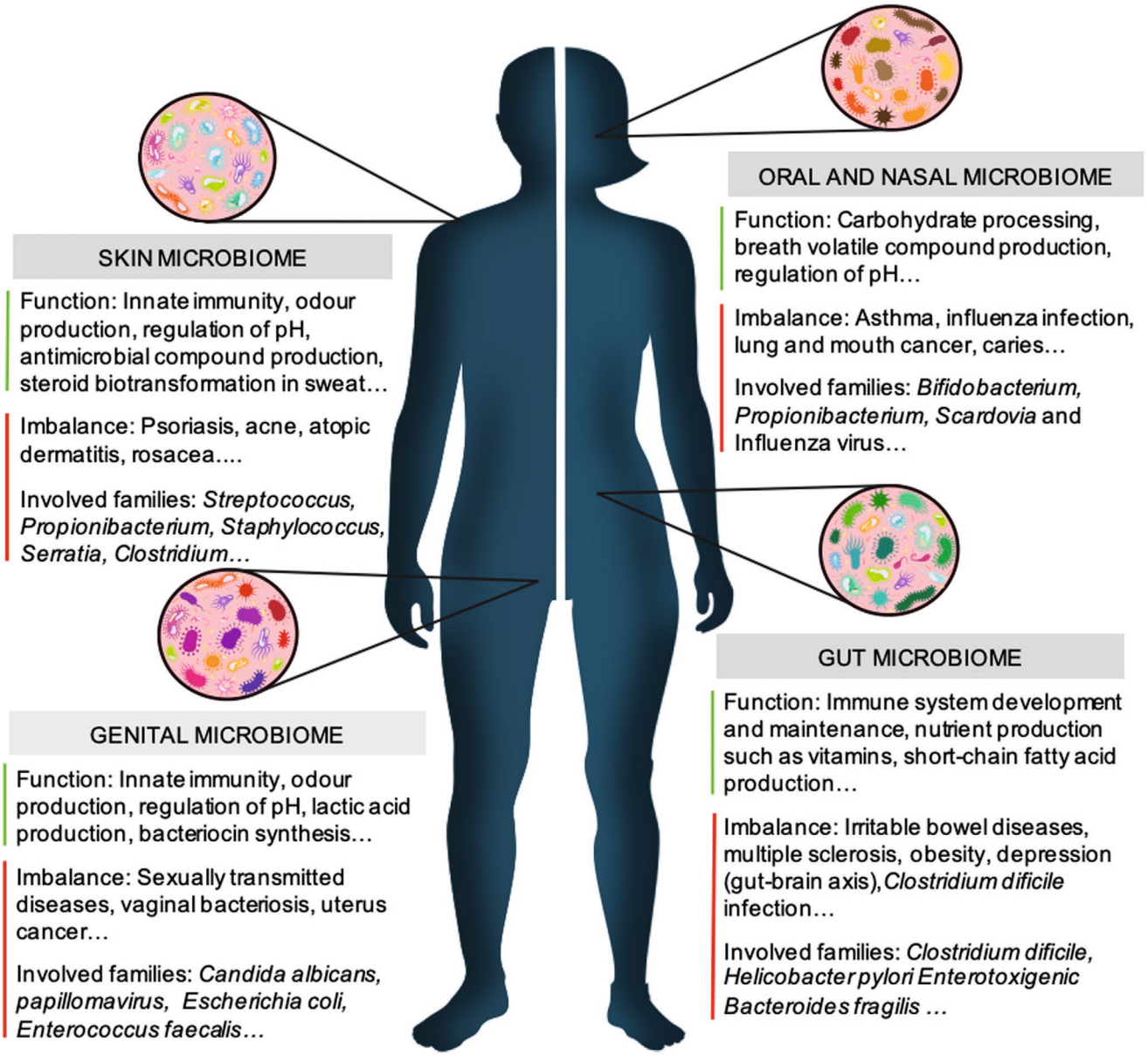


Figure 1. Simplified overview of commensal microbes by body location, showing their corresponding functions and links to human health. Imbalances in local microbial populations have been linked to myriad diseases. Some examples of involved bacteria in these conditions are shown.

drug use).[6,7] Such factors modulate the number and relative abundance of microbiome species, known as the alpha diversity. Based on this premise, El Rakaiby et al. referred to the human microbiome as "a cloud of genetic information accessory to the stable human genome".[8]

1.2. Clinical Impact

The continuous communication among humans, their microbiome and their environment largely dictates short-term variability in alpha diversity, which precludes definition of a stable or default microbiome corresponding to good health.[8] For instance, one study found that alteration of the gut-flora composition by non-conventional gut bacteria provokes secretion of the antibacterial peptides alpha defensins by host innate immune cells, and another found that conventional gut flora induce Paneth cells of the small intestine to express the antibacterial protein angiogenin-4, which targets gram-positive bacteria.[9,10] Despite a certain degree of transitory variability, which itself varies among humans, researchers have demonstrated that for roughly 60% of the species in an individual's microbiome, the population remains constant over a 5-year period.[11–13] Accordingly, the corresponding population data can be used as reference values, such that any significant changes in the relative or absolute population of one or more species could indicate a pathologic event.

Researchers have already associated alterations in the microbiome to various diseases (e.g., colorectal cancer, celiac disease, cirrhosis and inflammatory bowel disease), conditions (e.g., obesity and allergies) and clinical parameters (e.g., longevity).[14–18] For instance, strengthening of the natural commensal microbiota has been found to play a significant role in the immune response towards influenza virus.[19] Eventually, monitorization and use of specific probiotics might be very useful for the assessment of the diagnosis and prognosis of virus infections, as in the current SARS-CoV-2 outbreak.[20–24] Another example involves the microbiome gut-brain axis (Figure 1), whose constant communication pathway between nervous and endocrine systems modulates our behavior and our mental state (e.g., causing or preventing anxiety and depression).[16] At last, by re-examining whole-genome and whole-transcriptome sequencing studies in The Cancer Genome Atlas (TCGA) of 33 types of cancer from treatment-naive patients, Poore et al. have found unique microbial signatures in tissue and blood within and between most major types of cancer, suggesting that these microbial nucleic acids could have a diagnostic value.[25] Thus, due to all the foreseeable implications the microbiome has on human health, our group and many others consider that monitoring of the microbiome, beyond its value for fundamental scientific research, represents a novel diagnostic strategy for personalized medicine.

1.3. Microbiome R&D: From Technology to Signatures of Diagnostic Significance

Over the past few decades, the tools available to study the microbiome have become increasingly sophisticated and diverse, from the first culture-based and DNA-sequencing techniques (the 1970s), to next-generation sequencing (the early 2000s), to the latest omics-based approaches.[26] For example, previously, using culture-based techniques, researchers could isolate only 30% of the species of the gut microbiota; in contrast, omics-based approaches have revealed a nearly complete view of the alpha diversity.[27–30] The current gold-standard technique for studying the human microbiome is metagenomics, which enables discrimination of bacterial and archaeal DNA from host DNA, based on measurement of prokaryote-specific genes known as 16S ribosomal RNA genes. Metagenomics results enable taxonomic classification of microbiota at a given point in time, which can then be used to generate a profile of alpha diversity to facilitate comparison of different microbial samples: for instance, from the same individual at different times, or from distinct individuals or cohorts.[31]

Correlating alpha diversity with a given condition or disease is not trivial, chiefly because not all the bacteria present at a given time are implicated in the condition or disease in question.[32] For instance, a detected species might be only transiently present when the sample is collected, or the cells may even be dead but are still detected. This limitation has been partially offset in the past 5 years, through the use of techniques such as metatranscriptomics (RNA), metaproteomics (proteins), and metabolomics (metabolites), which provide information on different levels of distinct molecules within a population of different bacterial species.[27] The results are then compared with metagenomic studies to find a relation to microbiota composition, whereby dead or transient species can be ruled out. Thus, the combination of metagenomics with metatranscriptomics, metaproteomics and metabolomics generates a multi-omics approach that can give a much better understanding of specific microbiome behavior.[27]

As emphasized by Petrosino et al., numerous studies have now revealed microbiome–disease associations, making the microbiome a key component of precision medicine.[33] This is for example the case in the fields of inflammatory bowel diseases; liver diseases such as cirrhosis, rheumatic diseases such as arthritis, oncology and response to anticancer chemotherapies.[25,34–38] In another example, Pedersen et al. have shown that the serum metabolome of insulin-resistant individuals is characterized by increased concentrations of branched-chain amino acids, which correlate to a gut microbiome having an enriched biosynthetic potential for these acids.[39] Integrating all this information is important to understand how the microbiome interacts with the host in response to changes in the microbial population, in diet, in aging or in health or disease. Thus, the diagnostic question has evolved from simply determining the presence or absence of concrete species in the microbiome, to characterizing how certain species relate to each other, to the host and to health or disease.

Although there are a large number of studies mainly based on metagenomic analyses, a review of the literature reveals a growing number of works that are produced by integrating multi-omic analyses. These studies can make it possible to document the links between microbiome and pathologies. For example, Franzosa et al. performed untargeted metabolomic and shotgun metagenomic profiling of cross-sectional stool samples from discovery and validation cohorts of Crohn's disease, ulcerative colitis and control patients.[34] By integrating metagenomics and metabolomics data, they identified 122 robust associations between differentially abundant species and well-characterized differentially abundant metabolites, thus suggesting potential diagnostic and therapeutic targets.[34] In the same line, Price et al. followed 132 subjects (with Crohn's disease, with ulcerative colitis, or without inflammatory bowel disease, i.e., controls) for one year each to generate integrated longitudinal molecular profiles of host and microbial activity during disease (up to 24 time points each; in total 2965 stool, biopsy, and blood specimens).[35] They observed a characteristic increase in facultative anaerobes at the expense of obligate anaerobes, as well as molecular disruptions in microbial transcription (for example, among Clostridia), metabolite pools (acylcarnitines, bile acids, and short-chain fatty acids), and levels of antibodies in host serum.[35] Of course, the translation of these findings in diagnostic tools will require comparative analyses that will be enabled by data access through data repositories, and also further validation studies.

Multi-omic analyses have also the potential to shed light on the mechanisms of interaction between the microbiota and the host. This has for example been achieved by Rosser et al. who highlighted the role of microbiota-derived metabolites to suppress arthritis by working on samples obtained from patients and an animal model.[37] They showed that stool butyrate levels are reduced in patients with rheumatoid arthritis compared to healthy controls, and that supplementation with

Table 1. Microbiome related studies on therapeutics, diagnostics, and involving (bio)sensors as an indicative of the growing trend of these biosensors in clinical trials.

Number of microbiome studies in clinicaltrials.gov (25. October 2020)	Total number	Completed	Ongoing (recruiting)	Terminated/ withdrawn/ suspended
Therapeutics	1023	285	499	51
Diagnostics	370	72	198	16
Involving Sensors/biosensors	16	4	12	0

butyrate suppresses arthritis severity in a mouse model. They also demonstrated that butyrate increases the concentration of 5-hydroxyindole acetic acid, a metabolite derived from serotonin that activate aryl-hydrocarbon receptor in regulatory B cells.[37]

Microbiome derived signature can also have a diagnostic value, as emphasized by the study performed by Oh et al., who identified diagnostic signatures for fibrosis from stool metagenomic and metabolomic profiling that, when combined with serum aspartate transaminase levels, distinguishes cirrhosis in mixed fibrosis cohort.[36] The cohort included 163 participants encompassing non-NAFLD (non-alcoholic fatty liver disease) controls, NAFLD-cirrhosis patients, and their first-degree relatives. The gut metagenomic and metabolomics signatures comprised 19 discriminatory species (among the 310 microbial species identified) and 17 metabolites (among the 435 quantified ones) that accurately detected NAFLD-cirrhosis in the proband cohort, respectively.

The growing interest in the human microbiome as a determinant in health and disease, the creation of new scientific consortia, and increasing participation in studies by members of the public (see Section 1.3.1 and 1.3.2), together set the stage for the human microbiome to be included in precision and personalized medicine.

1.3.1. The Microbiome Diagnostics Market and Current Products

The microbiome therapeutics & diagnostics market is expected to grow from $506 million in 2022 to $899 million by 2025 (a compound annual growth rate of representing an 22.1%).[138] This prediction suggests growing acceptance of microbiome-based products by the general public. This market can be divided into two segments: therapeutics and diagnostics. Although both are slated for expansion, diagnostics are expected to grow the most, due mainly to the discovery of microbiome-related biomarkers for oncology.

Presently, there are commercially available microbiome-related tests and analyses; however, these products are not recommended for use alone as diagnostics. Today's analyses, such as uBiome, analyze the alpha diversity of the microbiome at one or more timepoints, to enable temporal monitoring of microbiome composition and correlation of it to life events. These results can be also analyzed by a healthcare provider to help to determine a diagnosis. A simpler technology is provided by Evivo, which produces a PoC test to screen newborn babies for the commensal bacterium *Bifidobacteria infantis*. Newborns acquire their gut microbiome during passage through the birth canal, which that inoculates the baby with *Bifidobacterium,* protecting it from harmful bacteria and serving as the basis for the remaining microbiome. The Evivo test relies on a change in fecal pH when the gut is first colonized by this bacterium; thus, results are quickly obtained for babies suffering from low levels of *Bifidobacterium*. Another interesting product with potential for microbiome diagnostics is MinIon (Oxford Nanopore Technologies), a portable, real-time DNA/RNA sequencer. Although this device was not designed for microbiome analysis per se, it can process microbiome samples and classify the microbiota by phyla and genera. It reads electrical signals that are generated by the different nucleotides when passing through a nanopore. It enables in situ sequencing, de novo sequencing, targeted sequencing, metagenomics studies, and epigenetics studies, amongst others. Nevertheless, MinIon is only intended for trained users.

1.3.2. International Consortia

Many international projects have been launched to characterize the human microbiome and analyze its role in human health and disease. In 2007, researchers in the Human Microbiome Project, funded by the National Institutes of Health (NIH), used metagenomics to study commensal microbiota in healthy individuals at five anatomic locations: the nasal cavity, the oral cavity, the skin, the gastrointestinal tract and the urogenital tract.[139] After they were unable to define a standard healthy microbiome, NIH researchers are now exploring host-microbiome interplay in three clinical scenarios: pregnancy, inflammatory bowel disease and diabetes.[140] In the MetaHIT project, which ended in 2012, researchers studied the intestinal tract microbiota of 1000 healthy people, ultimately dividing the cohort into three classes, or enteromes, defined by the dominant bacterium in their respective microbiota.[141] Other platforms, including the Human Food Project and the Earth Microbiome Project, joined together to create the American Gut Project (AGP), an open research platform that accepts microbiota samples from anyone in the world.[142] They aim to characterize the taxonomic and functional diversity of the microbiome and its diversity across human populations to define a baseline healthy microbiome.

Several international projects already employ multi-omics approaches.[142] For example, by using metagenomics and metabolomics, researchers in the open-source project AGP seek to correlate participants' samples with clinical parameters (e.g., age) and lifestyle parameters (e.g., dietary, smoking, or drinking habits).

Our groups are currently developing a nanobiosensor for detection and monitoring of biomarkers for cirrhosis and acute-on-chronic liver failure (ACLF), as part of the European consortium Microb-Predict, which is integrating multi-omics data from over 10 000 subjects from different cohorts to develop novel diagnostics and treatment strategies for these conditions. Specifically, we aim to create a diagnostic platform to detect ACLF at an earlier stage than presently possible, by monitoring the patient's microbiome.

1.4. Clinical Trials Landscape in the Interface of Microbiome and Nanotechnology

The gut microbiome may serve as biomarker for disease progression, severity and treatment response.[40] Indeed, diagnostic

biomarkers deriving from the gut microbiome have been tested in different diseases, such as diabetes, colorectal cancer, and cirrhosis.[36,41–45] Microbiome may also determine the treatment response and thereby guide patients management, as shown for epithelial cancer and melanoma, where the use of immune therapy is a move towards targeting the microenvironment by employing nanotechnology.[46–50] Therefore, intervention on the microbiome are being extensively explored as indicated by more than 1000 registered trials, with half of them ongoing (Table 1).

Yet, analyzing the gut microbiome is not always easy, since also the location of the microbiome is important as shown in gastroenterology and hepatology.[51,52] Patients with different diseases exhibit both disease-specific changes, as well as non-specific shared responses in their gut microbiome.[53] Therefore, one should first identify robust disease-specific signatures, and even more relevant the mapping of the microbiome changes during the specific disease, which should be independent of other confounding factors. This is still difficult to foresee, since the confounding effect size of different known technical and biological factors ranges between 10%–15%.[40] Moreover, large-scale longitudinal data from across the world are required to account for the confounding factors, since the variation in the genus composition of gut microbiome by the specific diagnosis may change only by including or removing confounders, such as alcohol consumption or diet. Another major confounder for the gut microbiome composition are drugs, and not only antibiotic, but also non-antibiotic drugs, one in four of which may change at least one gut species and this is likely underestimated.[54] Some of the non-antibiotic drugs may induce even bacterial resistance, while others may improve diversity.[54,55]

Having said this, it is not surprising that a large number of studies have used and are currently using the microbiome for diagnostic purposes (Table 1). Through the revolution in the microbiome field, different options are now available to identify diseases in microbiome profiles, as shown recently by the Microbiome Search Engine.[56] Moreover, integration with other omics data may facilitate that the dream of "personalized" medicine becomes reality.[33]

Still robust gut microbiome biomarkers are feasible, as demonstrated recently for colorectal cancer across large geographic distances and different studies, but the applicability requires the expertise of nanotechnology.[44] In fact, there are very few studies using sensors and biosensors to analyze the microbiome (see Table 1). Especially in these studies, none of them is testing or validating a sensor for the detection of the microbiome, but those detecting glucose, volatiles, sleep, movement, heart rate variability and endotoxin levels. The field of microbiome currently urges for easy-to-use tools in the determination of the diagnostic and prognostic signatures, and nanotechnology may develop the answer.

2. The Need for New PoC Devices and New Sensors

The future of precision and personalized medicine would require the access to in-depth routine analyses that provide the maximum information possible. In order to include the human microbiome, some aspects need to be considered.

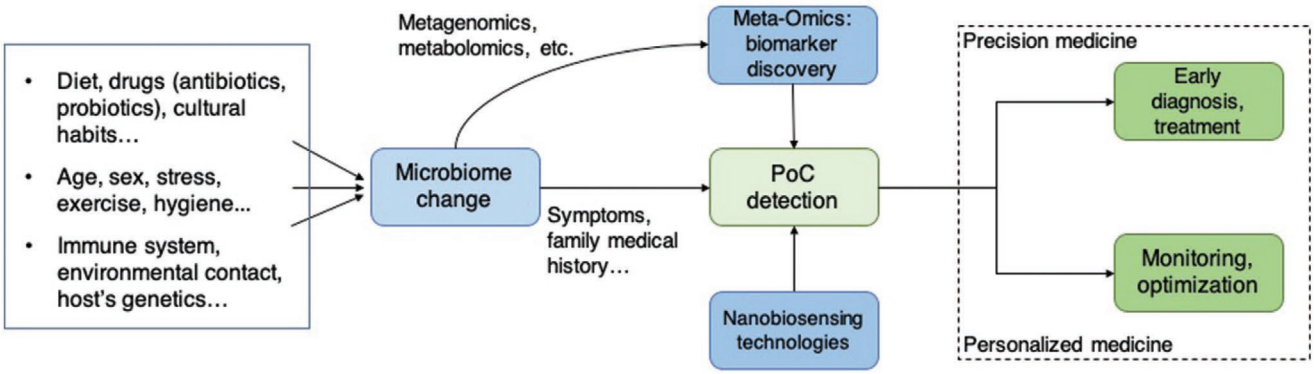
a. As previously mentioned, obtaining a list of species and metabolites contained in a particular microbiome by means of metagenomics or other omics is not entirely useful. There still exists the need of analyzing the results and relating them to the patient's lifestyle, which is an exhaustive work. Besides, it is unlikely that healthcare centers could implement routine metagenomic analyses due to the high cost of the equipment and the highly trained personnel required.
b. Nowadays routine analyses regarding microbiological samples rely on culture-based approaches, which are slow and only provide information about few species, and not metabolites.

Instead, future precision and personalized medicine should establish a panel of biomarkers for each microbiome-related condition in order to simplify and fasten routine analyses. These biomarkers would include cells, nucleic acids and other molecules, and the technology used for their detection should adapt to a convenient format in order to bring these analyses to the everyday life with a low cost.

We think that point-of-care (PoC) nanobiosensors would be suitable for this purpose. Importantly, the World Health Organization has established criteria to facilitate access to, and use of, new PoC devices by all types of patients in developing and developed countries, known collectively as REASSURED (Real-time connectivity, Ease of specimen collection, Affordability, Sensitivity, Specificity, User-friendliness, Rapid and Robust operation, Equipment-free and Deliverability).[57] Thus, any new PoC device for microbiome-based diagnostics should ideally adhere to the REASSURED criteria. Within the realm of PoC sensors, nanobiosensors harness the properties of nanomaterials (e.g., higher surface area, their optical and electrical properties and their high tunability) to achieve superior analytical and usability performances.[58–60] Considering the unique characteristics of nanomaterials is of great importance when orchestrating the detection of the very diverse biomarkers involved in the microbiome, such as cells, lipids, proteins, nucleic acids and small molecules.

The huge amount of data generated by multi-omics approaches has enabled discovery of microbiome biomarkers associated with specific conditions and diseases, which in turn are providing a window to new novel diagnostics strategies.[70] Thus, one could envision development of sensitive and fast sensors for hospital- or home-based monitoring of microbiome-related biomarkers, which could ultimately change diagnosis and daily life for patients suffering from various diseases, similarly to how glucometers have changed the lives of diabetic patients (see **Scheme 1**).

We believe that nanobiosensor-based PoC devices for detection of biomarkers could replace the current gold-standard techniques of metagenomics and culture-based analyses, which are expensive and time-consuming. Thus, we review here nanobiosensors that respond to the more general definition of PoC devices, meaning that they can provide results wherever the patient needs them (e.g., at home or at the hospital). However,



Scheme 1. Nanobiosensing for detection of validated microbiome-related biomarkers based on multi-omics data. Point-of-care devices based on nanobiosensors could enable prevention, early diagnosis and monitoring of microbiome-related conditions according to diverse clinical parameters (e.g., symptoms, antibiotics use, age, family medical history) and lifestyle parameters (e.g., diet, stress, or drug use).

due to the variety of microbiome-related biomarkers, ranging from small molecules to whole bacteria (see **Table 2**), and to their complicated analysis, which involves special equipment or specific sample treatment methods, meeting REASSURED criteria is not always feasible.[57] Therefore, we also discuss nanotechnologies that we refer to here as "quasi- PoC", that might require to be used in a laboratory or to be interpreted by trained personnel. Although these devices or technologies cannot be used at home or other locations directly by patients, they could be implemented in healthcare centers for efficient, cost-effective, routine testing.

3. PoC Nanobiosensors and Microbiome-Related Biomarkers

As the microbiome is constantly changing, PoC sensors for microbiome-biomarkers should comply with extra monitoring and multiplexing capabilities. In fact, obtaining clinically-relevant information on a patient's status may require simultaneous, real-time monitoring of multiple biomarkers such as metabolites, sugars, lipids, nucleic acids, peptides and proteins.[71] Additionally, PoC sensors must overcome the challenges faced by other biosensors: namely,

Table 2. Examples of microbiome-related biomarkers and availability of dedicated (nano)biosensors for the diagnosis of different diseases. The type of molecule and weight/size column serves to illustrate on the very different kinds of potential biomarkers. mRNA: messenger RNA.

PoC	Technology	Disease	Biomarker	Type of molecule	Weight/Size	Sample	Ref
Already exists	Lateral flow	Intestinal inflammation	Calprotectin	Zinc-binding protein	35.6 kDa	Stool	[61]
Already exists	Lateral flow	SARS-CoV-2	Human IgG/IgM	Immunoglobulin	150 kDa	Saliva	[23]
Optimizable	Lateral flow	Tuberculosis	Mycobacterial lipoarabinomannan (LAM)	Lipoglycan	17.4 kDa	Urine	[62,63]
Optimizable	Paper-based sensor	*Clostridium difficile* infection (CDI)	Calprotectin mRNA	Nucleic acid	–	Stool	[64]
			CXCL5 mRNA		–		
			IL-8 mRNA		–		
Not available	–	Equine grass sickness (EGS)	4-cresyl-sulfate	Organic compound	188.2 kDa	Urine	[65]
			Hippurate		179.2 kDa		
			TMAO		75.1 kDa		
			O-acetyl carnitine		203.2 kDa		
Not available	–	Colorectal cancer (CRC)	Matrix-metalloproteinase-9 (MMP-9)	Enzyme	92 kDa	Stool	[66]
Not available	–	Multiple sclerosis	Lipid 654	Lipopeptide	653.5 kDa	Stool	[67]
Not available	–	Lung cancer	Capnocytophaga	Nucleic acid	0.3–0.5 µm	Saliva	[68]
			Veillonella	Whole cell	0.3–0.5 µm		
Not available	–	Cirrhosis	Kynurenic acid/Quinolinic acid	Metabolites	<500 Da	Plasma	[69]
Not available		Cirrhosis	Molecular signature including 17 discriminatory metabolites	Metabolites	<500 Da	Stool	[36]
			Molecular signature related to 19 discriminatory bacterial species	Nucleic acid sequences			

that small-molecule biomarkers present steric hindrance caused by the congestion of the molecules by its surrounding ligands, and that complex media can pose challenges in terms of specificity and ease-of-use. In this context, nanomaterials offer a great opportunity to provide novel PoC sensors with the required performance. Here, we describe the advantages of nanomaterials as the basis of PoC sensors for detection of microbiome-related biomarkers. These include relatively large surface areas, easy functionalization, and unique optical and electrical properties.

3.1. Lateral-Flow Assays

3.1.1. Overview of Lateral-Flow Assays

Most PoC nanobiosensors are based on lateral-flow assays (LFAs), the best-known of which is the home pregnancy test, which detects or measures the hormone human chorionic gonadotropin (hCG) in urine. Given their ease-of-use, LFAs have been extended for detection or measurement of various analytes such as toxins, pathogens, pesticides, metal ions, drugs and proteins.[58–60] Importantly, LFAs meet nearly all the REASSURED criteria;[57] however, their sensitivity remains limited. A typical LFA comprises a paper strip onto which a liquid sample is added, which generates a chemical reaction whose result is discernable by the naked eye (e.g., by color change). Specifically, the sample moves via capillary action through different zones of the strip, each of which is dedicated to a specific function (e.g., sample loading and target labelling; migration and analyte detection; and sample pumping). These assays employ signal amplifiers conjugated to bioreceptors (e.g., antibodies, nucleic acids or enzymes), which enable recognition of the target analyte(s) (**Figure 2**A).[60]

3.1.2. Nanomaterials and LFAs

Many LFAs exploit the unique optical properties of nanomaterials. Specifically gold nanoparticles (AuNPs) are the most used label in LFAs, thanks to their easy functionalization and plasmon properties that confer them a strong red color that is ideal for naked eye detection.[58] As alternative to AuNPs, iridium oxide nanoparticles, dyed latex beads, and carbon nanoparticles and nanotubes have also been employed as colorimetric labels in LFA.[60,76] In order to improve the LFA sensitivity yet using standard AuNPs, some developers have also taken advantage of their catalytic properties, used them as carrier for enzymes, and used their quenching properties in fluorescence-based measurements.[77–79]

Instead, applications requiring a higher degree of sensitivity often rely on fluorescence signals. In this arena the most used nanoparticles are semiconducting or carbon-based quantum dots, silica beads and liposomes (Figure 2B).[58,80,81] Comparing them to conventional organic dyes, their higher quantum yield and stability make them ideal labels for PoC applications, although they do require a fluorescence reader. Another useful quality of quantum dots is the possibility to excite them with a UV light and record different emission spectra depending on the type of quantum dot employed, making them ideal labels for the multiplexed detection of biomarkers including different mycotoxins, food contaminants and viruses.[58,80,81] Finally, more recently, research groups employed upconverting nanoparticle (UPTs) to achieve even greater sensitivity, although we must point out that their high cost may prevent their use at a large scale.[82]

Beyond optical detection, nanomaterials serve other roles in LFAs. For example, they have been used as signal generators to improve sensitivity in diverse readout strategies (using chromophores and fluorescent dyes) such as surface-enhanced Raman spectroscopy (SERS) over the usual or standard LFA.[79,83–85] Intriguingly, other different nanomaterials can be combined to enhance sensitivity, as has been shown through use of graphene to quench quantum dots as a revealing agent in LFAs for detection of bacteria, or the use of magnetic nanoparticles for preconcentration and purification of the sample.[86–90]

3.1.3. LFA Challenges

These and many other examples suggest the utility of LFAs for simple, microbiome-related applications. However, there remain major challenges to this end. Firstly, multiple detection of different types of molecules is complicated due to the use of bioreceptors of different natures under a single measurement and conditions. Secondly, microbiome analyses often require simultaneous detection of more than ten analytes (for which we saw the possible use of quantum dots as multi-color labels), which is not trivial in terms of sensor fabrication and sensor readout, even when using a smartphone. Thirdly, analyses of microbial nucleic acids often entail detection at extremely low concentrations (copy numbers), which would demand an integrated amplification step or the use of ultra-sensitive labels (e.g., UPTs). Similarly, microbiome analyses may require detection of protein analytes in very low ranges, like the femtomolar range, which is still difficult to achieve.

3.2. Electrochemical Sensors

3.2.1. Overview of Electrochemical Sensors

Another technology used for PoC diagnostic devices is electrochemical sensing, as in the widely successful example of the glucometer and much beyond. Although electrochemical PoC sensors require a reader, making them less portable than LFA-based PoC sensors, their superior analytical performance compared to the latter (greater sensitivity, quantitative measurements and real-time monitoring) is ideal for specific applications, such as monitoring of glucose levels.[91,92] The most common electrochemical techniques used in PoC sensors are cyclic voltammetry,[93–95] differential pulse voltammetry,[91] chronoamperometry,[96,97] electrochemical impedance spectroscopy[98,99] and field-effect transistor (FET)-based sensing.[100,101] The first three techniques are used to analyze the currents obtained in response to fluctuations in the concentration of a redox reporter, which can be quantitatively related to the concentration of the target analyte.[102] In contrast, impedance

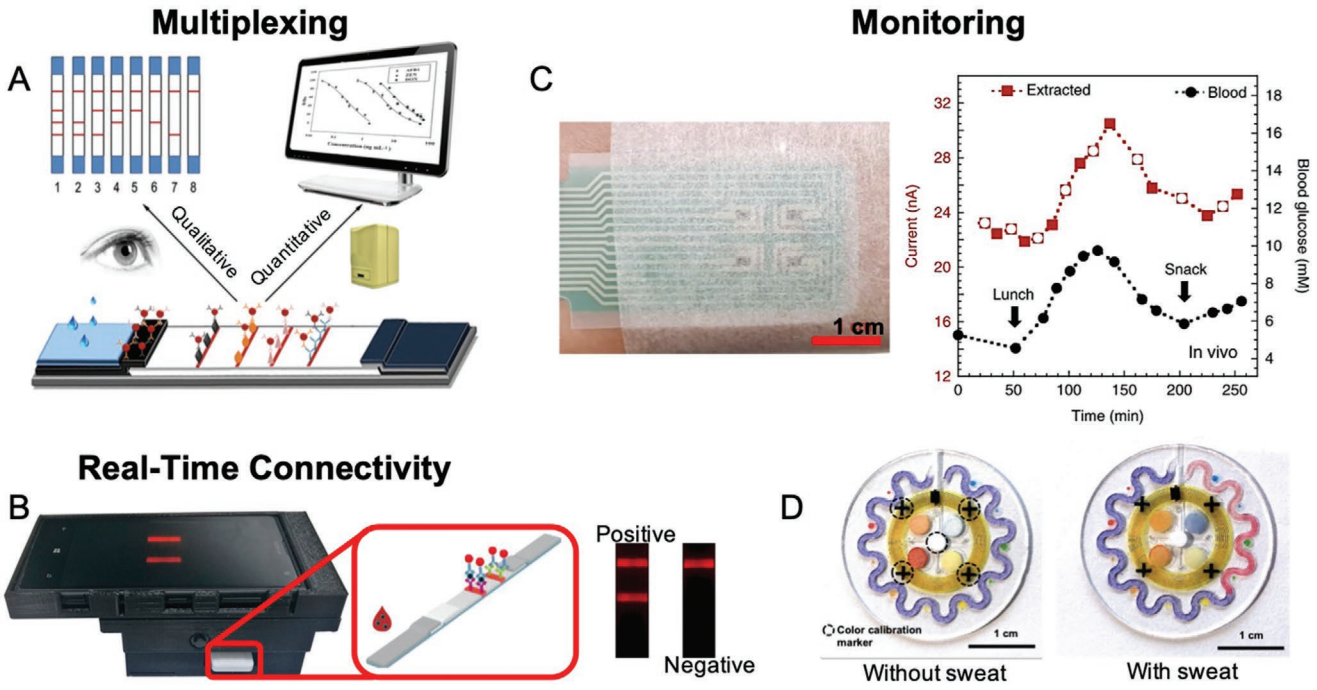


Figure 2. Point-of-care biosensing devices amenable to microbiome analyses. A) Multiplexing: Schematic of a multiplex lateral-flow immunoassay (LFA) for multiple mycotoxins. Adapted with permission.[72] Copyright 2014, American Chemical Society. B) Real-time connectivity: Smartphone-based fluorescent LFIA platform for highly sensitive PoC detection of Zika virus non-structural protein 1. Reproduced with permission.[73] Copyright 2019, Elsevier. C) Monitoring (C&D): In-vivo continuous glucose monitoring in healthy human subjects. Left: screen-printed array fixed onto a volunteer's forearm and connected to a potentiostat. Right: values of interstitial fluid-borne glucose as read from extracted blood (red) or through the skin (black). Adapted with permission.[74] Copyright 2018, The Authors, published by Springer Nature. D) Epidermal microfluidic sweat-monitoring device before (left) and after (right) injection of artificial sweat. Lactate, glucose, creatinine, pH, and chloride ions in the sweat sample are monitored and integrated near-field communication (NFC) electronics enabled smartphone-based analysis. Reproduced with permission.[75] Copyright 2016, AAAS.

spectroscopy and FET-based sensing detect the analyte according to changes in the electronic properties of the sensor surface induced upon specific binding of the analyte to it.

3.2.2. Nanomaterials and Electrochemistry

As with LFA-based sensors, analytical performance in electrochemical sensors has been optimized through strategic use of nanomaterials, either as transducers in the sensing platform or as electrochemical reporters. Examples of such transducers include electrodes fabricated from (or modified with) AuNPs, silver nanoparticles (AgNPs), carbon nanotubes, graphene or magnetic nanoparticles (Figure 2C, **Figure 3**A,B).[91,103] Specifically, AuNPs and AgNPs have been widely used as transducer components in electrochemical assays due to their high conductivity and easy functionalization. Furthermore their high stability and homogenous distribution (achieved using simple synthesis protocols such as the Turkevich method)[104] allow for the fabrication of electrochemical sensors using easy and low-cost methods such as screen or inkjet printing.[91,105–107]

More recently, researchers have been working on exploiting the high electron mobility and capacity of 2D nanomaterials such as reduced graphene oxide and molybdenum disulfide to enhance electrodes' conductivity and analytical performance.[108,109] Since its discovery in 2004,[110] graphene has attracted considerable attention owing to its remarkable properties, including high carrier mobility and capacity, an ultrathin form factor, an ambipolar field effect, and highly tunable conductance.[111,112] Graphene has been extensively explored in the development of high sensitivity biosensors based on FETs (gFETs), by using the resistivity variation in the gFETs due to the binding of the target molecule to the bioreceptor on its surface.[101,113]

Transition metal dichalcogenides, 2D layered materials with ultrathin structures such as molybdenium disulfide is attracting a lot of interest too. This semiconductor has a tunable bandgap and piezoelectric properties, which allow to improve the sensitivity of these sensors and make them more stable.[114] The intercalation of specific elements in the structure of these materials allows precision functionalization, lowering its performance dependence by environmental variables. Moreover, the ultrathin structure of these materials gives them excellent mechanical properties such as flexibility and strain resistance, both very sought characteristics for wearable biosensors for instance.[115] However, the limits of these materials are usually bound to their synthesis and to the production of a truly uniform nanosheet able to completely cover the sensor's electrodes. Consequently, these types of sensors keep costs relatively high with respect to the standard ones.

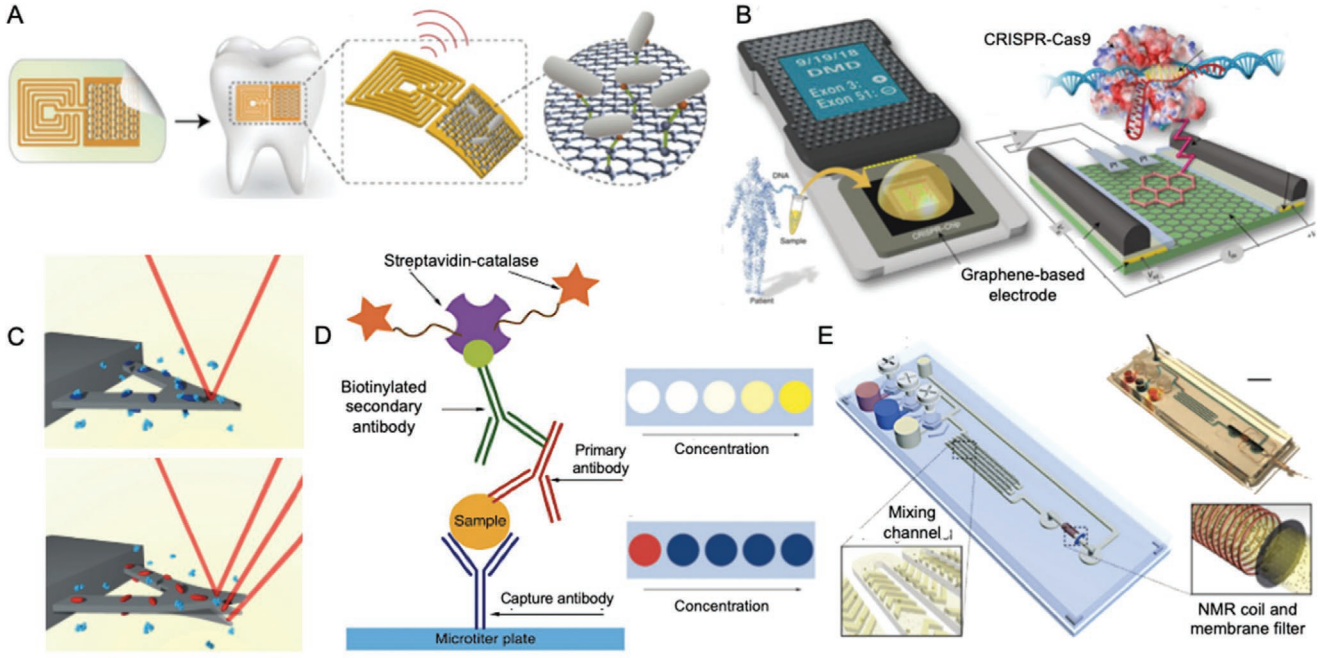


Figure 3. Examples of nanobiosensing technologies with potential for microbiome-related diagnostics. A) Wireless, biotransferrable, graphene-based nanosensor (left). Graphene is printed onto bioresorbable silk and contacts are formed containing an inductive resonant coil for wireless transmission (middle). Magnified schematic of the sensing element, illustrating wireless readout of the binding of pathogenic bacteria by peptides self-assembled on the graphene nanotransducer (right). Adapted with permission.[118] Copyright 2012, Springer Nature. B) CRISPR-enhanced, graphene-based, field-effect transistor (CRISPR–Chip), which enables facile, rapid and selective detection of a target sequence contained within whole intact genomic DNA. Adapted with permission.[101] Copyright 2019, The Authors, published by Springer Nature. C) Illustration of interactions between bacterial target and probe molecules on cantilever beam. Specific biomolecular interactions between target and probe molecules alter the intermolecular nanomechanical interactions within a self-assembled monolayer on one side of a cantilever beam. Adapted with permission.[119] Copyright 2013, Springer Nature. D) A conventional, colorimetric, enzyme-linked immunosorbent assay for visual detection of the target antigen (top, right). A non-conventional colorimetric ELISA or Plasmonic ELISA, whose ultra sensitivity allows the detection of the target at ultra-low concentrations with the naked eye (down, right). Adapted with permission.[120] Copyright 2012, Springer Nature. E) Highly sensitive, magnetic barcode assay for detection of tuberculosis, equipped with polymerase chain reaction (PCR) chambers, mixing channels, and a microcoil for NMR measurements. The PCR-amplified target genes from Mycobacterium tuberculosis are captured and magnetically labeled by a pair of complementary oligonucleotides conjugated to microspheres and magnetic nanoprobes. Adapted with permission.[121] Copyright 2013, Springer Nature.

Looking at the use of nanomaterials as electrochemical reporters there are three main characteristics that must be considered: size, functionalization and signal generation. Overall a successful electrochemical label should have a size in the same scale as the one of the bioreceptor (mostly in the low nm range), a surface chemistry that allows for an oriented functionalization and the ability to generate a strong electrochemical signal. All these aspects are generally found in nanoparticles, rather than 2D materials. Overall, AuNPs and AgNPs are the most used electrochemical labels in voltammetric and amperometric sensors. Specifically, the former measure the voltammetric peak of Au and Ag, while the latter rely on their catalytic activities, such as the hydrogen evolution reaction of AuNPs. As for LFA, also electrochemical sensors can employ QDs in multiplexed sensors, thanks to the different voltammetric peaks related to the different QD composition.[116]

3.2.3. Electrochemical Sensors Challenges

Several of the commonly recognized advantages of LFA sensors, such as sample filtration and purification, amplification strategies, and reagents mixing can be implemented also within electrochemical sensors thanks to the use of similar substartes as for the electrochemical paper-based analytical devices (ePADs) and of nanomaterials. What is still a challenge is the immediacy of a LFA result if compared to the need of a readout instrument and the interferences of electrochemically active compounds in the sample which may alter the result of the tests if not filtered or without signal normalization. With respect to the readout instrument, smartphones are becoming an interesting platform for the purpose, considering how widespread they are. Conversely, with reference to interference, big testing campaign and common interference compensation in the sample of interest remain the only weapons against it.

Interestingly, in the context of microbiome-related diseases (e.g., Crohn's disease), wearable electrochemical devices can be used to monitor inflammation and other physiological symptoms (Figs. 2C & 3A).[117] Considering the nearly infinite number of possible combinations of functionalized nanomaterials, electrochemical PoC devices could, in theory, be used to detect a broad array of microbiome-related biomarkers.

4. Quasi-PoC Nanobiosensors with Potential in Microbiome-Related Diagnostics

Recent advances in nanotechnology have enabled conception of exciting new diagnostic sensors that, despite not meeting REASSURED criteria,[57] should provide a basis on which to construct future PoC devices or at least, devices for hospital or laboratory use. Below, we highlight representative devices with strong potential for detection of microbiome-related biomarkers.

4.1. Cantilevers

Microcantilevers (or microelectromechanical systems) comprise a cantilever structure that measures the vibration or deflection generated by the adsorption of a molecule on its surface. Known for their high sensitivity, these devices can detect analytes in the picomolar to femtomolar range (Figure 3C).[122,123] Nanocantilevers (cantilevers of thickness from 10 nm to 90 nm) exhibit even better limits of detection and require less sample. For example, researchers have demonstrated that use of nanocantilevers to detect specific DNA sequences may obviate the DNA-amplification step required by microcantilevers and other methods.[122] Nanocantilevers have already demonstrated good performance at determining antibiotic resistance in *Escherichia coli*.[119] Accordingly, nanocantilevers could be used to detect nucleic acids that indicate microbiome-related skin condition (e.g., atopic dermatitis), as they are present at only low concentrations in skin-related samples.[124–126] However, since microcantilevers and nanocantilevers are very fragile structures, their portability is currently limited, thus precluding their use in PoC devices. Nevertheless, they can still be used as bench-top tools in laboratories and healthcare centers.

4.2. Plasmonic Sensors

Plasmonic sensors based on plasmonic nanomaterials have been used in various biosensing strategies. Here, we present two different types of plasmonic sensors: surface plasmon resonance (SPR) biosensors and plasmonic enzyme-linked immunosorbent assays (ELISAs).

Surface plasmon resonance is a physical phenomenon that occurs when free electrons (or surface plasmons) at the interface of a conductive material and another medium oscillate when focused by polarized light. These oscillations are very sensitive to surface changes, making SPR sensors ideal for label-free detection of molecular-binding events (e.g., antibody/antigen binding). Nanomaterials such as AuNPs, AgNPs and magnetic nanoparticles have been the most reported, mainly due to their high degree of modification and high refractive index.[127] For instance, Kaur et al. developed a highly sensitive, highly specific ZnO/Au-based SPR biosensor for rapid diagnosis of bacterial meningitis—a condition that can be fatal if not diagnosed sufficiently early—based on detection of *Neisseria meningitidis* DNA.[128] Similarly, Dey et al. developed an SPR biosensor for detection and quantification of *E. coli* cells in blood plasma.[129] Their device requires minimal operational expertise, making it highly amenable to PoC applications. However, despite this example and other widespread efforts to bring SPR sensors into PoC scenarios, these devices tend to be expensive and require specialized equipment.[129,130] If researchers can reduce the costs and resolve the matrix-effect issues caused by interfering components of human samples when using SPR biosensors, then these devices could be implemented in laboratories and healthcare centers for faster and more detailed routine analyses.

ELISA tests are nowadays a common tool in hospitals and healthcare centers as in vitro screening diagnostic tests. Plasmonic ELISAs exploit the innate plasmonic properties of gold nanoparticles, properties which can be tuned during nanoparticle synthesis. For instance, De la Rica et al. managed to use the output of ELISA to control both the synthesis of AuNPs and the growth of gold nanostars.[130,131] Thanks to the strong and sensitive optical signal generated by the plasmon of those particles, they obtained outstanding detection limits (in the range of 10^{-21} M) with the naked eye (Figure 3D).[130] Thus, plasmonic ELISAs could be used to detect microbiome-related proteins at small concentrations, especially those protein biomarkers that are very scarce in biological samples. The major drawbacks of plasmonic ELISAs are their multi-step protocols and requirement for trained personnel, making them best suited for laboratories and healthcare centers. However, it is envisaged its integration along with smartphones and microfluidic devices for its use in in-field measurements of human fluids such as urine, blood or plasma.[132–134]

5. Future Perspectives

Here, we outline the three main features that we believe are essential for any PoC sensor used for microbiome-related diagnostics: multiplexing, smartphone connectivity, and automation (for sample processing).

Personalized medicine often entails the need to quantitatively detect multiple biomarkers for a given disease. Multiplexing capability on a test is especially desirable for microbiome-related diseases, as mentioned above. In fact, depending on the relative abundance of each microorganism or biomarker in question, different diagnosis might be reached (see, for example: Figures 2A and 2D). Furthermore, considering the ever-increasing number of microbiome-related biomarkers that are being discovered, we envision that nanobiosensors focused on the microbiome could address multiple diseases and conditions within a single test. Researchers developing nanobiosensors will have to face this problem and devise smart solutions for the quantification of multiple biomarkers, possibly even of different molecular classes.

Widespread adoption of smartphones in developing and developed countries alike provides an ideal opportunity for PoC devices based on nanobiosensors, either through direct integration or indirect coupling. Replacing complicated external equipment with patients' or caregivers' phones would be a massive advance towards meeting the REASSURED criteria. Smartphones enable real-time connectivity, are user-friendly and provide imaging and processing power sufficient for clinically relevant sensing performance. Interestingly, some PoC devices

(see, for example: Figure 2D) already include near-field communication (NFC) technology, which enables users to exchange data between a device and a smartphone. This is especially useful for monitoring, as it enables visualization of temporal changes in biomarker levels and correlation of such changes with life events. Moreover, smartphones can convert qualitative measurements (e.g., those from LFAs or plasmonic biosensors) into semi-quantitative measurements, and translate the complex readout signals of multiplexed sensors into more comprehensible results (see Figure 2B).[132]

Finally, given that detection of microbiome-related biomarkers involves treatment of very small samples from diverse matrices (e.g., blood, saliva, urine and stool), we feel that automated processing of samples within diagnostic devices would greatly facilitate PoC use. This can be done with lab-on-a-chip technology. For instance, samples can be sequentially treated (preconcentrated or purified) and sensed through the combined use of microfluidic channels with functional modules such as pumps, filters, actuators and sensors.[135–137] An example is shown in Figure 3E, which depicts a tuberculosis diagnostics device that detects *Mycobacterium tuberculosis* DNA in human sputum samples.[121] It comprises a PCR chamber for amplification of sample DNA; mixing chambers; and a microcoil for nuclear magnetic resonance (NMR) measurements, equipped with highly sensitive magnetic nanoparticles for enhanced NMR quantification. Microfluidics can also be applied for monitoring purposes. For instance, Koh et al. (Figure 2D) developed a wearable device for analysis of glucose, chloride, lactate and pH levels in sweat, which can be coupled to a smartphone.[75] The technologies underpinning these examples could eventually be combined with one or more of the countless nanomaterials now available, to provide PoC devices for continuous monitoring of specific microbiome-related biomarkers. Such devices, when used by the patient at home, would make repeat visits to laboratories or healthcare centers unnecessary. However, automated sample processing for PoC devices continues to be limited by reproducibility issues and the historic need for on-board pumps.

5.1 Ethical, Legal, and Societal Issues Pertaining to Human Microbiome R&D and Innovation

Growing debate over the human microbiome mirrors increasing promotion of personalized medicine, which prioritizes R&D and innovation as well as projects mean to improve quality of life for citizens. Exploring the ethical, legal, and societal issues related to human microbiome R&D and innovation requires consideration of all pertinent local, national, European and International laws and guidelines.

From the ethical and legal perspectives, human microbiome samples should be treated as other human biological samples. Thus, in general terms and with some exceptions, human microbiome samples could be used for research purposes but only upon informed consent of the owners (healthy volunteers or patients). These considerations should also apply to regulation of biobanking of human microbiome samples. Accordingly, human-microbiome R&D should be reviewed by independent and multidisciplinary research ethics committees, to assess any potential scientific, technical, ethical, legal, or societal concerns.[143]

Human microbiome samples and their associated personal data pose an ethical and legal challenge to protect individuals and to preserve their privacy and the confidentiality. Thus, this area of research is covered by the European General Data Protection Regulation (GDPR) as well as by the Convention on Biomedicine and Human Rights of the Council of Europe, which prohibits genetic discrimination.[144,145] Human microbiome samples should not be subject to due diligence obligations under EU Regulation 511/2014, as states have no sovereign rights over human microbiome samples or over their citizens.[146,147] Thus, the sole owners of these samples are the individuals (healthy volunteers or patients) from whom they were obtained.

From a societal perspective, there is a need to raise awareness about the value of human microbiome R&D, to educate the public with accurate scientific evidence and to combat misinformation. This in turn requires researchers to reflect on their priorities—namely, in terms of how the outcome of their research could benefit society at large. The general public should have a basic understanding of the importance of the human microbiome and of how to assess the quality of testing and monitoring devices and services before accessing these devices and services.

6. Conclusions

Diagnosis of microbiome-related conditions through discovery, detection and measurement of appropriate biomarkers will be a cornerstone of future precision medicine. Here, we have shown that the field of nanobiosensing offers ample opportunities for future devices for such diagnostics, although many challenges remain in engineering PoC devices that meet the REASSURED criteria. Each of the nanomaterials and nanobiosensing technologies that we have covered here—including LFAs, electrochemical sensors, nanocantilevers, SPR biosensors, plasmonic ELISAs and microfluidics—presents a unique combination of advantages and disadvantages for use in PoC devices. Furthermore, the quality of the newly discovered biomarkers, and the analytical performance and robustness of nanobiosensors regardless of underlying technology, all remain to be improved. We believe that the nanobiosensing community has a responsibility to patients to seize this opportunity by designing, developing and facilitating the commercialization of new nanobiosensors for microbiome-based diagnostics, especially for PoC applications.

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Conflict of Interest

The authors declare no conflict of interest.

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